IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Matthew Ashby
Application No.	:	10/607,077
Filed	:	June 25, 2003
Confirmation No.	:	1068
Examiner	:	Teresa E. Strzelecka
Group Art Unit	:	1637
For	:	METHODS FOR THE SURVEY AND GENETIC ANALYSIS OF POPULATIONS

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Declaration of Matthew Ashby Under 37 CFR 1.132

Sir:

I, MATTHEW ASHBY of Mill Valley, California, hereby declare as follows:

1. I am the inventor of the above-mentioned application. I am an Adjunct Professor at the Romberg Tiburon Center for Environmental Studies at San Francisco State University, as well as President and Chief Scientific Officer at Taxon Biosciences. I received my Ph.D. in Biological Chemistry from the University of California at Los Angeles School of Medicine and

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my Bachelor's degree in Biochemistry and Cell Biology from the University of California at San Diego.

2. I am aware that the U.S. Patent and Trademark Office has rejected claims 45-50 and 57 of the above-identified patent application as allegedly failing to comply with the enablement requirement of 35 U.S.C. §112. In that context, I have read the July 14, 2008 Office Action.

3. I make this declaration to report the results of experiments performed under my supervision and control.

4. In sum, we generated short 16S ribosomal RNA (rRNA) tags from environmental samples (i.e., soil samples gathered from multiple locations) utilizing Serial Analysis of Ribosomal DNA (SARD), as described in Example 1 of this application, and identified tags whose abundance correlate with geochemical parameters (e.g. metal and oil deposits) in these samples, using the methodology described in this application. We then used the tag sequences to identify counterpart 16S rRNA gene clones, designed PCR primers for these genes, and used quantitative PCR to quantitate the abundance of the genes in a different and larger set of environmental samples to prospect for the geochemical parameter. What follows is a more detailed discussion of the experiments we conducted and the results we obtained.

5. We generated 1,664 non-identical short 16S ribosomal RNA (rRNA) tags from 21 different soil samples using SARD. To identify specific SARD tags whose abundance correlates with geochemical parameters, we conducted a correlation analysis, as described in the specification of the above-identified application, e.g., at page 24, line 7 to page 30, line 1, and in Figures 6, 9 and 11. First, within each sample, we clustered the SARD tags according to how well the abundance of each tag correlated to the abundance of other tags. This results in groupings of tags that tend to be found together in the same samples. Second, we clustered the samples in the second dimension based

on similar tag composition. By following this procedure, we generated a 2-Dimensional hierarchical cluster analysis of the abundance of the tags (*see* Exhibit A). Each row represents a distinct SARD tag (characterized by its nucleic acid sequence), and each column represents a different soil sample. The intensity (black to white) represents the relative abundance of each SARD tag in a given soil sample. We then compared geochemical data (taken from the same soil samples) with the SARD tag distributions. The comparison revealed correlations between clusters of SARD tags and certain geochemical parameters, such as copper and aluminum (*see* Exhibits A1 and A2, respectively). Exhibit A1 shows a group of 13 SARD tags (selected out of 1,664 tags) whose abundance in the samples correlated with the abundance of copper in the same samples. That is, samples that tend to have a greater abundance of these 13 SARD tags also tend to have more copper. Exhibit A2 shows a different group of 10 SARD tags whose abundance correlated with the abundance of aluminum.

6. To identify organisms that are represented by the copper-correlated SARD tags, we successfully recovered longer fragments of the 16S rRNA gene clones from which the copper-correlated SARD tag sequences had presumably originated. These longer sequences were identified by screening the DNA sequences of a collection of 16S rRNA gene clones (derived from the same samples) for sequences with a perfect match to the nucleic acid sequence of the SARD tags of interest. These 16S rRNA genes were thus the likely source for the SARD tag that had demonstrated the correlation of interest. Comparison of the 16S rRNA gene clone recovered from one of the copper-correlated SARD tags revealed that this sequence was from a member of the β-Proteobacteria division and is likely a member of the *Burkholderia* genus. Interestingly, three close relatives of this species have known associations with copper (e.g. they are stimulated by low levels of copper and resistant to high levels). This observation supports the mechanistic association of the identified tag sequence with copper, and is independent of our original quantitative analysis of the SARD tags.

7. Following the procedure described above in paragraph 6, six 16S rRNA gene clones were recovered from one of the tags that showed an especially strong correlation to aluminum (r^2>0.99). These gene sequences were found to be from the Acidobacteria division and were strongly associated with members of the Acidobacteriaceae family. This result is interesting, since there are no known metabolic roles for aluminum, and shows the power of the methods this invention.

8. The experiments and results described above are a direct continuation of the experiments described in Example III of the above-identified application, and use the specific teachings in the application. In fact, the first two columns of the array shown in Exhibit A are the samples Wy-1 and Wy-2 discussed in Example III.

9. As described in the application, once 16S rRNA gene sequences that show statistically significant associations with a given geochemical parameter are identified, they are useful as nucleic acid marker sequences, or "bioindicators" in a quantitative PCR (qPCR) assay. An implementation of the qPCR assay strategy is described below for another set of tags and parameters correlated with them.

10. Subsurface accumulations of oil and natural gas often leak low molecular weight hydrocarbons, such as propane, that migrate vertically and create low concentration plumes on the surface. Therefore, the abundance of propane bioindicators should also correlate to the presence of subsurface accumulations of oil and natural gas and should be useful as a prospecting tool for such resources. We generated a large collection of 16S rRNA tags from soil samples collected from Texas and California oil and gas fields using SARD, and identified tags that correlate to the presence of propane through 2-Dimensional hierarchical cluster analysis. This analysis was done using the procedure discussed above in paragraph 5, and disclosed in the above-identified application, e.g., at page 24, line 7 to page 30, line 1, and in figures 6, 9 and 11.

11. We used the propane-correlated SARD tag sequences to recover longer fragments of the corresponding 16S rRNA genes (using the procedure described in paragraph 6), and designed PCR primers. We then performed qPCR using six different primer pairs to quantitate the abundance of the six different 16S rRNA sequences (and hence, propane) in samples collected in a gridded array format over a known, producing gas field. The abundance of these bioindicators was interpolated between the sampling locations to create a contoured, color-coded map illustrating the location of propane plumes (Exhibit B). This exercise revealed a good concordance between the bioindicator-predicted location of propane plumes and the producing limits (as evidenced by the location of producing gas wells) of the field. Therefore, this quantitative map, generated in accordance with this invention, is useful for detecting the presence of subsurface oil and natural gas. In fact, as can be seen in Exhibit B, the existing gas wells tended to be located in the areas that had higher levels of the propane bioindicators. Thus, we confirmed that the abundance of the bioindicators, generated and used as described in this application, correlate to, and accurately prospect for and diagnose, geochemical parameters.

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Dated: 12-23-08

Signature: _____
Matthew Ashby

Exhibit A

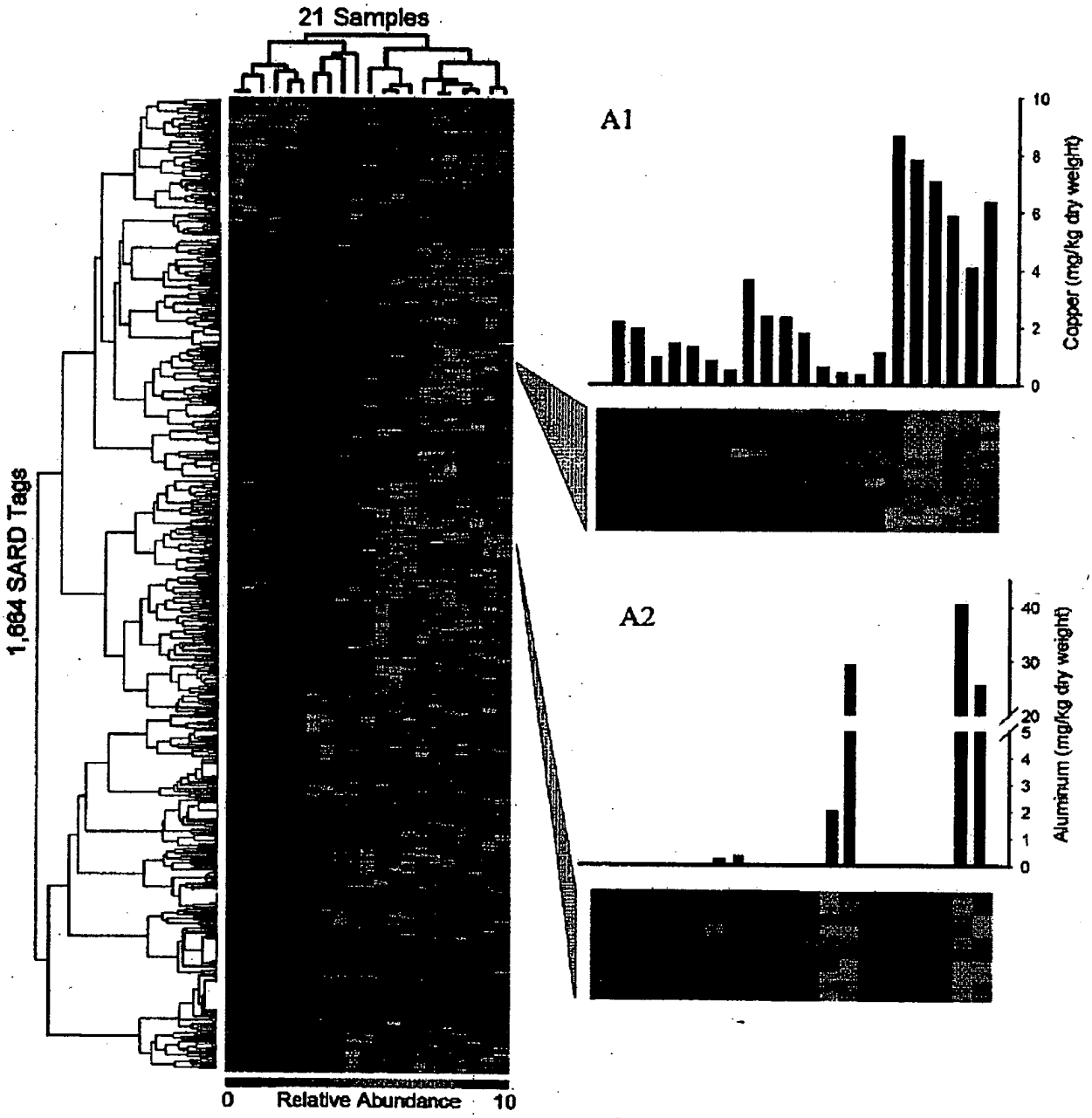

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Exhibit B

Quantitative Map of Bacterial DNA Sequences